Biologic and Structural Differences of Thrombopoietic Growth Factors

C. Glenn Begley and Russell L. Basser

The search for a thrombopoietic agent has resulted in the identification of numerous cytokines and growth factors with thrombopoietic activity. However, with the exception of interleukin (IL)-11 and thrombopoietin (TPO), the megakaryopoietic activity of most of these molecules has not produced clearly identifiable clinical benefits. Despite the relatively modest effect of IL-11 on megakaryocyte and platelet production in vitro and in vivo, it does reduce the need for platelet transfusions in specialized clinical settings. In contrast, the c-Mpl ligand TPO has been shown to be a potent stimulator of megakaryocyte and platelet production both in vitro and in vivo. Clinical studies are being conducted with two different preparations of the c-Mpl ligand: recombinant human thrombopoietin (rhTPO) and pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF). A recombinant form of the complete human molecule, rhTPO is glycosylated and produced in mammalian cells. PEG-rHuMGDF consists of only the receptor-binding domain linked to a polyethylene glycol (PEG) moiety and is generated in *Escherichia coli*. Although c-Mpl ligands are still being evaluated, preliminary evidence indicates that these molecules can elevate platelet counts and may be useful in a range of clinical contexts. This report discusses aspects of the biology behind the clinical actions of IL-11 and the c-Mpl ligands.

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PLATELETS ARE FUNDAMENTAL to hemostasis in the body. More than 90 years ago, Wright[1] first proposed that platelets were shed from megakaryocytes, which are themselves derived from progenitor and stem cells in the bone marrow. In recent years, major advances have been achieved in understanding the extracellular growth factors that are important in regulating this process. Consequently, a number of growth factors have been identified that are able to stimulate megakaryocyte and platelet formation.

The earliest in vitro and in vivo data that showed a direct action of defined hematopoietic growth factors on megakaryocytes came from studies with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-3.[2-4] Although both GM-CSF and IL-3 display a clear action on megakaryocytes, this effect has not translated into significant clinical benefit. Similarly, growth factors (such as stem-cell factor [SCF], or steel factor) and the lineage-specific molecules (erythropoietin and granulocyte colony-stimulating factor [G-CSF]) are active on megakaryocytes and their precursors, but this has not resulted in clearly identifiable clinical benefit. While IL-1 also has a stimulatory effect on megakaryocytes, its thrombopoietic action is probably indirect.[5,6]

The IL-6 family of molecules, another group of molecules that act on megakaryocytes and their precursors, has had more clinical success. These molecules all need the signal-transducing receptor molecule gp130. The molecules that possess megakaryopoietic activity within the IL-6 family include IL-6, IL-11, oncostatin M (OSM), and leukemia inhibitory factor (LIF). Despite the relatively modest effect of IL-11 on megakaryocyte and platelet production in vitro and in vivo, IL-11 reduces the need for platelet

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EXHIBIT 5

transfusions in specialized clinical settings. Additionally, the action of IL-11 and other members of this family on megakaryocyte precursors and on hematopoiesis appears to represent only a portion of the wide spectrum of IL-11 activities in many tissues.

In contrast, thrombopoietin (TPO) is a relatively "lineage-specific" cytokine that stimulates megakaryocyte production and maturation in vitro and is the most potent in vivo thrombopoietic growth factor identified to date. Gene-targeting studies have established that TPO is the most important physiologic regulator of steady-state megakaryocyte and platelet production.[7-9] Cloning of the c-Mpl ligand has led to the clinical development of two different preparations of TPO: recombinant human thrombopoietin (rhTPO) and pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF). The purpose of this review is to provide an understanding of the biology and key functional differences between these thrombopoietic factors.

Interleukin-11

IL-11 was originally identified as a stromal cell–derived growth factor that stimulated the proliferation of a murine plasmacytoma cell line.[10,11] However, the action of IL-11 on multiple hematopoietic lineages and its ability to stimulate the growth of multilineage hematopoietic progenitors were soon recognized.[12] Despite the clear evidence that IL-11 has multiple effects on hematopoietic cells, it is not essential for hematopoiesis either in the resting state or in response to hematopoietic stress. Mice that do not respond to IL-11 show normal blood indices, normal levels of progenitor cells, and normal functioning of the stem-cell compartment.[13,14] Furthermore, the effects of IL-11 are not restricted to hematopoiesis; it helps in recovery of spermatogenesis after chemotherapy,[15] ameliorates the effect of cytotoxic injury on the intestinal epithelium and inflammatory bowel disease,[16,17] and possibly mediates bronchial reactivity.[18] In addition to the multiple actions mediated by IL-11, gene-targeting experiments revealed that the critical action of

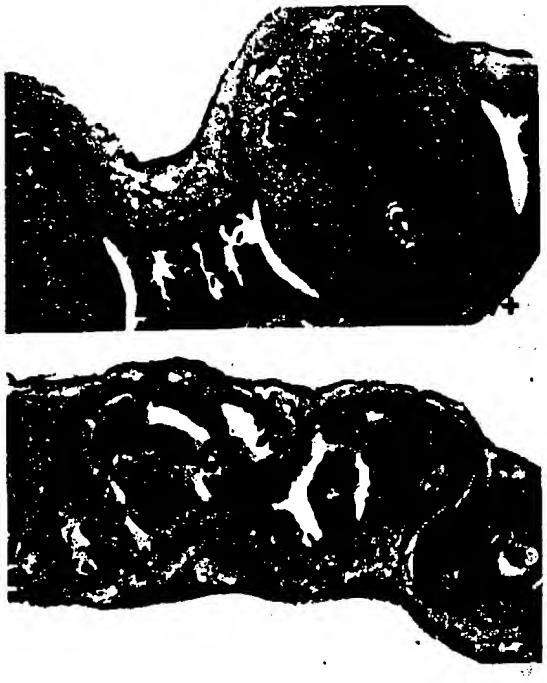

Figure 1. Decidual response of normal mice and IL-11 receptor null mice. IL-11 is crucial for maintenance of pregnancy. Top shows normal decidual response that occurs during embryonic development. The developing embryo is in the middle of the field, surrounded by a healthy, normal maternal decidual response within the uterus. Bottom shows the failure of decidual response when IL-11 responses are ablated in IL-11 receptor null (−/−) mice. The day of embryonic development (E) is indicated. Reprinted with permission from Robb et al.[19]

this molecule also involves an unexpected organ system. Female mice that lack the IL-11 receptor (IL-11Rα) are infertile because the uterine decidual response that normally follows implantation does not occur (Fig 1). Thus, the essential physiologic function of IL-11 is to maintain female fertility.[19]

The pleiotropic actions of IL-11 on the hematopoietic compartment and other organ systems are similar to those of other members of the IL-6 family of growth factors, including IL-6, LIF, OSM, ciliary neurotrophic factor, and cardiotrophin. Overlapping function of the IL-6 family of cytokines is, in part, explained by their shared use of glycoprotein (gp)130 as a signal transduction unit.[20,21] Several of these molecules also use the LIF receptor as a component f their receptor complex. In addition,

the growth factors IL-11, IL-6, OSM, and ciliary neurotrophic factor have specific receptor chains. IL-11Rα is widely expressed and confers low-affinity IL-11 binding. Coexpression with gp130 results in high-affinity binding of IL-11 and the capacity for signal transduction.[22,23]

IL-11 and Thrombopoiesis

IL-11 acting alone clearly has no megakaryocyte colony-stimulating activity in vitro, but it can augment the megakaryopoietic activity of cytokines such as IL-3, SCF, and TPO.[24-26] However, IL-11 alone and in synergy with IL-3 has induced features of megakaryocyte maturation, including increased megakaryocyte ploidy and size and production of acetylcholinesterase. Moreover, in vivo experiments with IL-11 found both enhancement of megakaryocyte progenitors and increased platelet production; in clinical studies, this action has been exploited for therapeutic gain.

Whether the action of IL-11 on megakaryocytes is direct or mediated via other factors has been controversial. Megakaryocytes have been shown to express IL-11 receptors, thus providing evidence for a direct effect of IL-11.[27] Similarly, researchers using highly purified target cell populations in an attempt to eliminate contaminating stromal elements that could act as a source of other factors found that IL-11 acting alone induced megakaryocyte maturation markers in purified human $CD41^+$ cells.[28] On the other hand, considerable evidence has been presented that the action of IL-11 on megakaryocytes might be indirect. For example, human megakaryocytes have produced and responded to IL-6[29]; thus, the action of IL-11 on purified megakaryocytes might have been mediated through IL-6. However, addition of anti–IL-6 antibody to cultures did not abrogate the response to IL-11.[28] As the considerable potential of TPO to support megakaryopoiesis became apparent, experiments were performed to examine the possibility that TPO might mediate the thrombopoietic response to other growth factors, including IL-11. Addition of soluble c-Mpl receptor as a TPO antagonist abrogated in vitro megakaryocyte colony formation induced by IL-6, IL-11, and SCF from unfractionated bone marrow cells. This suggested a dependence on TPO.[30] The generation of mice deficient in TPO or its receptor c-Mpl allowed this issue to be addressed; administration of IL-6, IL-11, SCF, or LIF to these mice resulted in a platelet increment,[7,8] thus demonstrating in vivo that these growth factors can act independently of TPO and its receptor.

Additional evidence for the action of IL-11 in megakaryocytopoiesis comes from the analysis of IL-11 in vivo. Administration of IL-11 to mice increased the platelet counts and was associated with an increment in the number of megakaryocytes and progenitors in bone marrow.[31,32] IL-11 also induced megakaryocyte maturation with an increase in megakaryocyte ploidy. Mice in which IL-11 expression was enforced showed a persistent elevation of platelets and increased megakaryocytes.[33-35] Despite the thrombopoietic effects of IL-11, platelet and megakaryocyte levels and platelet recovery are normal in mice that are unresponsive to IL-11. Moreover, ablation of IL-11 signaling in TPO-unresponsive (c-Mpl null) mice has no additional effect on the thrombocytopenia observed in these animals.[9] IL-11 also showed megakaryopoietic and thrombopoietic action in monkeys[36] and dogs.[37] Administration of IL-11 to humans causes a modest elevation of platelet count, increased megakaryocyte numbers with a higher fraction in cell cycle, and a shift to higher ploidy.[38-40] Although the adverse effects of IL-11 limit its thrombopoietic effect, IL-11 has been cleverly exploited to reduce the requirements for platelet transfusion during chemotherapy.

While the action of IL-11 to elevate platelets is clear, the data regarding the relationship between circulating levels of IL-11 and platelet/megakaryocyte mass are contentious. Elevated IL-11 levels were seen in patients with immune-mediated platelet destruction and resulting increased megakaryocyte mass.[41] In contrast, TPO levels are not elevated with immune-mediated platelet destruction.[42,43] IL-11 levels were also reportedly elevated in patients with thrombocytopenia and decreased megakaryocyte mass owing to myelosuppression, with a significant inverse correlation between IL-11 levels and platelet counts.[41] However, this finding has not been seen consistently.

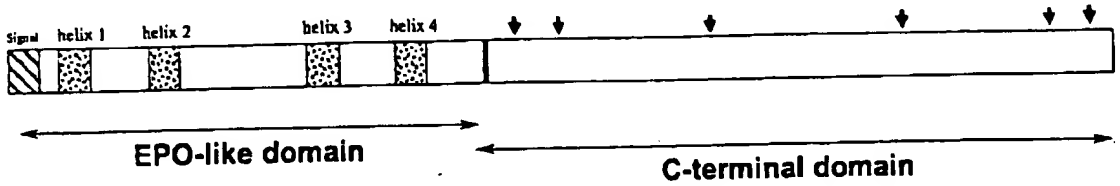

Figure 2. Domain structure of endogenous TPO. The amino terminus (first 153 amino acids) of TPO contains 4 conserved cysteine residues and is partly homologous to erythropoietin (EPO). Shaded boxes show the predicted α-helical regions of TPO. The carboxyl terminus (amino acids 154 to 332) of the molecule appears to be unique to TPO and contains the N-linked glycosylation sites indicated by solid arrows. Modified with permission from Foster and Hunt.[52]

Thus, unlike with TPO, there is no simple relationship between levels of serum IL-11 and total mass of cells of the megakaryocyte lineage. Consistent with the mouse studies mentioned above, no elevation of IL-11 levels has been observed in c-Mpl null mice with platelet counts 10% to 15% of normal.[8]

TPO: Principal Platelet Regulator

The molecular cloning of TPO was greeted with excitement, particularly because more than 40 years had elapsed since its existence was first suggested.[44] This molecule has been proposed as a differentiation factor during the final stages of megakaryocyte growth, leading to polyploidy, expression of platelet-specific proteins, and generation of platelets.[45,46]

As is often the case in research, several independent laboratories simultaneously reported the identification and molecular cloning of TPO.[47-51] A key event that preceded the molecular cloning of TPO was the recognition that the recently identified molecule, c-Mpl, was an excellent candidate for the TPO receptor.

The TPO molecule itself consists of two domains: (1) the receptor-binding domain that shows considerable homology to erythropoietin, and (2) the carbohydrate-rich carboxy-terminus of the protein, which is important in protein stability (Fig 2).[52] The availability of TPO cDNA clones allowed production of the recombinant TPO protein and the first real assessment of its properties. Clinical studies commenced very quickly using two preparations of the molecule (Fig 3). One, prepared by scientists at Genentech, Inc (San Francisco, CA), consists of the complete form of the human TPO molecule, or rhTPO. This preparation is produced in mammalian cells and is therefore glycosylated. The other preparation, prepared by scientists at Amgen Inc (Thousand Oaks,

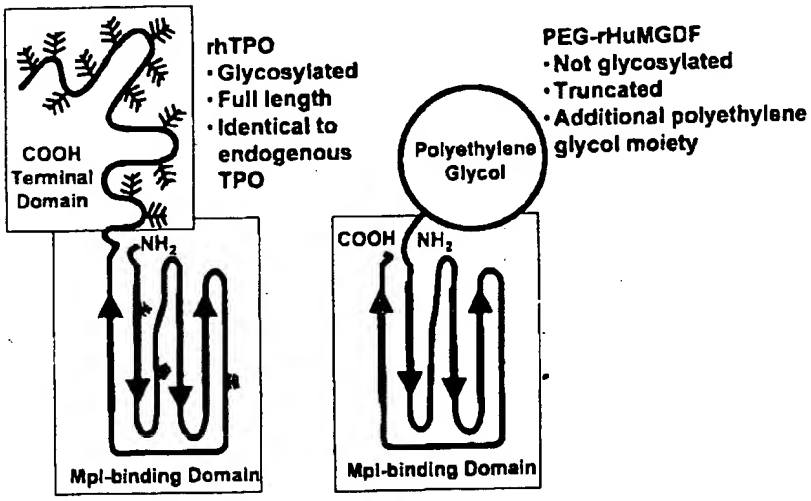

Figure 3. Molecular structures of rhTPO and PEG-rHuMGDF. Adapted with permission from Amgen, Inc (Thousand Oaks, CA).

Table 1. rhTPO Versus PEG-rHuMGDF: Pharmacologic Characteristics

	rhTPO	PEG-rHuMGDF
Terminal $t_{1/2}$	24-40 h	31 h
K_d for c-Mpl	150 pmol/L	150 pmol/L
Platelet clearance	1.28 mL/h/10^9 platelets	1.28 mL/h/10^9 platelets
Onset effect in mice	3 d	3 d
Peak effect in mice	4-6 d	4-6 d
Human route	Intravenous	Subcutaneous

Abbreviations: PEG-rHuMGDF, pegylated recombinant human megakaryocyte growth and development factor; rhTPO, recombinant human thrombopoietin; $t_{1/2}$, half life; K_d, dissociation constant.

CA), consists of the receptor-binding domain but not the carbohydrate-rich domain. The receptor-binding domain is conjugated to a polyethylene glycol moiety. This preparation retains all the biologic activity of TPO, is produced in *Escherichia coli,* and is referred to as PEG-rHuMGDF. As shown in Table 1, these preparations have similar pharmacologic characteristics and show dramatic in vitro and in vivo effects that translate clinically into an up to 10-fold increase in platelet counts. This is in contrast to the maximum twofold increase seen clinically with IL-11.

As predicted, TPO increases the size and number of megakaryocytes and stimulates the expression of platelet-specific markers and endomitosis in megakaryocytes in vitro. In addition, TPO is a potent megakaryocyte CSF, acting synergistically with other thrombopoietic cytokines. These crucial functions of TPO and its role as the principal physiologic regulator of platelet production have been confirmed in studies of mutant mice. These mice are either unable to produce TPO (TPO null) or unable to respond to TPO signaling (c-Mpl null). Consequently, they have a platelet count that is approximately 10% of normal and fewer megakaryocytes and their precursors.[53-55] A question remains regarding the factor(s) responsible for the residual platelets seen in these mice. To address this, double "knock-out" mice that lack the genes for c-Mpl and one other growth factor have been created. The researchers predicted that loss of IL-11 signaling, for example, would exacerbate the thrombocytopenia observed in mice lacking c-Mpl. However, mice that are doubly deficient in TPO and IL-3, IL-11, IL-6, or LIF have no additional defect in platelets or their precursors.[8,9] Thus, these growth factors alone are not responsible for the generation of platelets in the absence of TPO signaling.

In addition to the actions of TPO that were largely predicted, several effects were unexpected. First, the action of TPO is not lineage-restricted. Although TPO elevates the platelet count in patients without a detectable effect on peripheral white cell counts or erythroid parameters,[56-59] it is clearly able to act on these lineages. For example, when combined with factors such as erythropoietin or SCF, TPO is active on myeloid and erythroid precursors.[60-62] Consistent with this action of TPO on a broad spectrum of hematopoietic cells is its critical effect on the hematopoietic stem-cell compartment. This is perhaps the most surprising action of TPO. In the TPO null and c-Mpl null mutant mice, hematopoietic progenitor cells and stem cells are decreased, which reveals a crucial role for TPO in their development. Studies found that the number of multipotential hematopoietic progenitor cells in the bone marrow of c-Mpl null and TPO null mice was reduced by 50%.[7,55] Intriguingly, the defect in the progenitor cell compartment appeared to involve primarily the IL-11 responsive subset of precursor cells (unpublished data, June 1999). Preprogenitor cells and spleen colony-forming cells were reduced by 90%, and hematopoietic repopulating cells were deficient by an even greater magnitude.[63]

Other lines of evidence reinforce the important role of TPO signaling in early hematopoiesis. The c-Mpl is expressed on a subset of immature hematopoietic cells ($CD34^+$ cells). The viral homolog of c-Mpl (v-Mpl) induced an acute myeloproliferative leukemia in mice, with massive expansion of maturing cells of multiple

hematopoietic lineages. In other murine studies, in vivo administration of TPO expanded the bone marrow and spleen progenitor pools of all hematopoietic lineages.[64,65]

Another surprise was its relative inactivity of TPO on platelets and in the latest stages of megakaryocyte development. This is unlike the effects of G-CSF and GM-CSF, in which the action on mature myeloid cells is well established. Thus, although TPO can "prime" platelets, making them more responsive to agents like thrombin and collagen,[66-68] this priming does not cause any predisposition to platelet aggregation clinically nor any detectable adverse clinical consequences.[56,69] Perhaps the most significant consequence of this inactivity is the inability of TPO to hasten platelet shedding from megakaryocytes. In fact, if anything, TPO inhibits this process.[70] As a result, the action of TPO to elevate the platelet count is delayed, particularly when compared with the almost immediate effects of G-CSF on myeloid cells. The maximum elevation in platelet counts occurs 2 to 3 weeks after commencement of treatment with TPO. Therefore, TPO cannot meet a clinical need for immediate elevation of platelet count; TPO requires an expansion of the megakaryocyte-precursor compartment before the platelet count can increase. This intrinsic action of TPO has made its clinical development difficult. The delayed action of TPO results in a small window of opportunity during which stimulation of thrombopoiesis can overcome, for example, chemotherapy-induced thrombocytopenia.

An additional problem associated with the clinical development of PEG-rHuMGDF has been the creation of neutralizing antibodies to PEG-rHuMGDF, which cross-react with endogenous TPO. This has resulted in significant hematologic consequences in patients. In at least one case, it was associated with neutropenia and anemia as well as thrombocytopenia. This again presumably reflects the stem-cell action of TPO (unpublished data, January 1999). Although antibody formation has been seen with the subcutaneous administration of both forms of TPO and IL-11, the development of antibodies has not been reported to be associated with any adverse effects. The development of neutralizing antibodies to PEG-rHuMGDF is probably the consequence of subcutaneous administration; this problem can be avoided when the same molecule is administered intravenously (Shimosaka A, personal communication, July 1999), which is also the preferred route of administration of rhTPO.

Conclusion

Currently, IL-11 is the only molecule available clinically to elevate the platelet count after chemotherapy. Although clearly beneficial, IL-11 is associated with significant adverse effects. The clinical utility of two forms of TPO is currently being assessed. Both PEG-rHuMGDF and rhTPO show similar biologic function and are very effective in elevating platelet count. Their use, however, may be limited by the intrinsic biology of megakaryocytes. Although the clinical use of PEG-rHuMGDF has been limited by the development of neutralizing antibodies, rhTPO administration has not been associated with the formation of neutralizing anti-TPO antibodies. This difference may be due to the intravenous administration of rhTPO versus the subcutaneous administration used for PEG-rHuMGDF. Further clinical studies are needed to define the precise role and clinical benefit of these molecules.

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